Surfactin and Other Lipopeptides from *Bacillus* spp.

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Contents

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Abstract Isolated during 1950s and 1960s, the group of lipopeptides from *Bacillus* spp. gather more than 30 different peptides linked to various fatty acid chains. More than a 100 different compounds can so be described. In this chapter, they are classified into four main families: the surfactins, the iturins, the fengycins or plipastatins and the kurstakins. The biochemical mechanism responsible for their biosynthesis, which involved nonribosomal peptide synthetases, is described in detail. The complex cascade of regulation of surfactin synthetase operon and the environmental factors, which influence the lipopeptide production, are discussed. The main physico-chemical properties of these remarkable biosurfactants and their possible relationships with the biological activities are also presented. A brief overview of the molecular strategies developed to get modified lipopeptide compounds and the last bioprocesses set up for their production are given. In the last chapter, the main applications of surfactin are proposed.

1 Introduction: History of Lipopeptide Discovery in *Bacillus* spp.

In this section, we discuss a short history of the discovery of the various lipopeptides from different *Bacillus* species and the genes involved in their biosynthesis. Up to now, these lipopeptides were classified into three different families: surfactins, iturins and fengycins (or plipastatins). Following the discovery of kurstakins in 2000, we suggest that they should be added to the family of lipopeptides. A first overview of the different members of each lipopeptide family and the producing species is given.

1.1 Surfactins from Asia

In 1968, Arima et al. isolated an exocellular compound with an exceptional biosurfactant activity from the supernatant of a culture of *Bacillus subtilis*. This

compound was named surfactin and its structure was elucidated as that of a lipopeptide (Kakinuma et al. 1968). It was characterised as: a valuable inhibitor of fibrin clot formation, an antibacterial, antitumour and hypocholesterolemic agent. Other strains or species producing surfactin derivatives (*Bacillus coagulans,* Huszcza and Burczyk 2006, *Bacillus mycoides*, Athukorala et al. 2009) or related compounds, such as esperin (Thomas and Ito 1969), halobacillin isolated from a marine *Bacillus* strain (Trischman et al. 1994), lichenysin from *Bacillus licheniformis* (Horowitz et al. 1990), pumilacidin from *Bacillus pumilus* (Morikawa et al. 1992) or bamylocin A from *Bacillus amyloliquefaciens* (Lee et al. 2007), were also identified.

The *srfA* operon and the enzymes responsible for the biosynthesis of the surfactin were the first described for a lipopeptide from *Bacillus subtilis* (Nakano et al. 1991; Menkhaus et al. 1993). This discovery allowed for the confirmation that this lipopeptide is synthesised by the non-ribosomal pathway (see Sect. 3). The operon involved in lichenysin biosynthesis was then described by Konz et al. (1999).

1.2 Iturins from Africa

Mycosubtilin was the first antifungal lipopeptide from *Bacillus subtilis* mentioned in literature in 1949 (Walton and Woodruff). A second similar compound named iturin was described by Delcambe (1950). Its name is related to the Ituri, a region from Congo where the compound was isolated from a soil sample. Iturin was first characterised as a strong antifungal agent with a restricted antibacterial activity against *Micrococcus* and *Sarcina* strains. The precise structure of these two compounds and similar compounds from the same species was described during the 1970s and 1980s: mycosubtilin (Peypoux et al. 1976, 1986), bacillomycin L (Besson et al. 1977) identical to bacillomycin Lc or bacillopeptin (Eshita et al. 1995; Volpon et al. 2007), iturin A (Peypoux 1978), iturin C (Peypoux et al. 1978), bacillomycins D (Peypoux et al. 1981) and F (Peypoux et al. 1985). Production of iturinic compounds was also identified in other species such as: *Paenibacillus koreensis* (Chung et al. 2000), *Bacillus amyloliquefaciens* (Yu et al. 2002) and *Bacillus pumilus* (Cho et al. 2009). Mycocerein, an antifungal peptide produced by *Bacillus cereus* was partially described and could belong to the iturin family (Wakayama et al. 1984).

The genes involved in the biosynthesis of iturinic compounds have been first characterised for mycosubtilin in *Bacillus subtilis* ATCC 6633 (Duitman et al. 1999) and then for iturin A (Tsuge et al. 2001a) and bacillomycin D (Moyne et al. 2004; Koumoutsi et al. 2004).

1.3 Concomitant Discovery of Fengycin and Plipastatin

In 1986, German (Vanittanakom et al. 1986) and Japanese teams (Nishikiori et al. 1986) discovered simultaneously a third family of lipopeptides: fengycin produced by *Bacillus subtilis* and plipastatin from *Bacillus cereus*. The first lipopeptide was determined as an antifungal agent and the other as a phospholipase A2 inhibitor. Only small structural differences exist between these two compounds and a doubt still exists today about them as well as the differences in their biological activities. Production of fengycin was also demonstrated by *Bacillus thuringiensis* (Kim et al. 2004).

The operon encoding fengycin or plipastatin synthetases was first described in *Bacillus subtilis* 168 in 1997 (Tosato et al. 1997) and then in *Bacillus subtilis* b213 and A1/3 in 1999 (Steller et al. 1999) and in *Bacillus amyloliquefaciens* FZB42 in 2004 (Koumoutsi et al. 2004).

1.4 Kurstakins, a New Family of Lipopeptides from Bacillus spp.

The use of Matrix-assisted laser desorption/ionisation (MALDI) to characterise and differentiate between different species or strains of *Bacillus* spores allowed Hathout et al. (2000) to discover a new family of lipopeptides produced by *Bacillus thuringiensis* subsp. *kurstaki* and consequently called kurstakin. These compounds display antifungal activity against *Stachybotrys charatum* and are mainly adsorbed to the spore surfaces. Additionally, the potential operon encoding kurstakin synthetases was recently discovered (Bumpus et al. 2009; Abderrahmani et al. 2010).

1.5 Conclusion

Since the discovery of iturins, more than 30 different lipopeptides produced by different strains of *Bacillus* spp. have been characterised revealing the high potential of lipopeptide biosynthesis of this genus. The exponentially growing number of genome sequences should probably allow to discover new families or new variants in the near future.

2 A High Diversity of Structures

This section is dedicated to the description of the structure of the different lipopeptides. The different variants of each family are described in detail. Fig. 1 shows a detailed structure of two compounds of each family. Table 1 gives an overview of the different peptide sequences and lipidic moiety of the different variants described in each family.

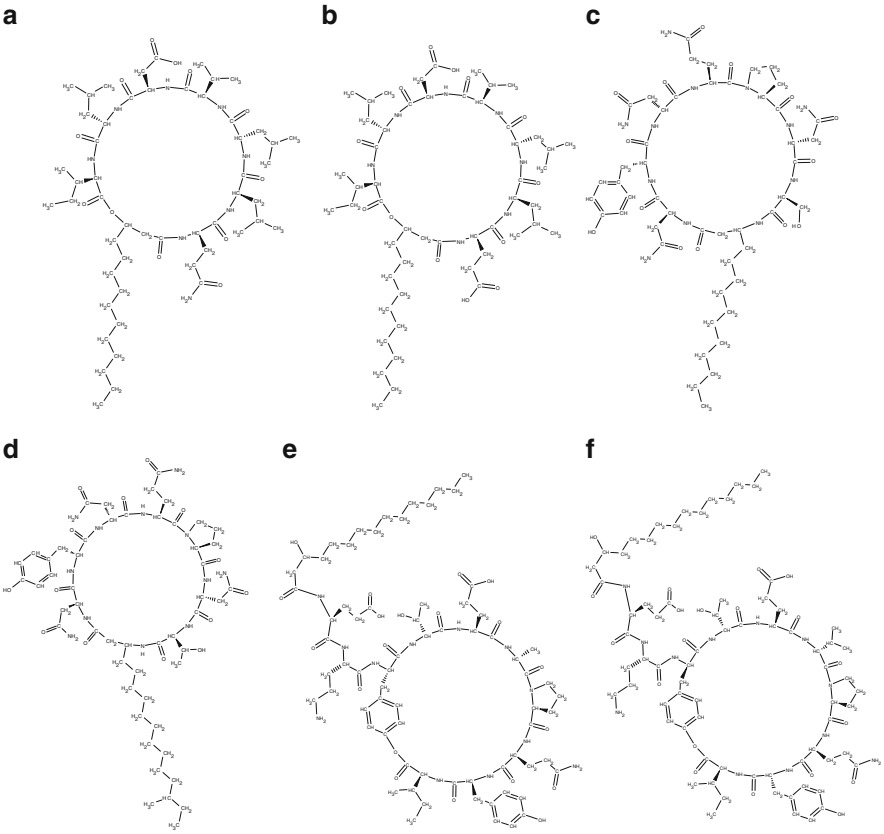

Fig. 1 Detailed structure of different lipopeptides (**a**) Lichenysin *n*C14 (**b**) Surfactin *n*C14 (**c**) Iturin *n*C14 (**d**) Bacillomycin F *a*C17 (**e**) Fengycin/plipastatin A *n*C15 (**f**) Fengycin/plipastatin B *n*C15

2.1 Surfactin and Related Compounds

The family of surfactin is composed of about 20 different lipopeptides (Bonmatin et al. 2003). With the exception of esperin (Thomas and Ito 1969), they have the common following structural traits: a heptapeptide with a chiral sequence LLDLLDL interlinked with a β-hydroxy fatty acid and with a D-Leu in position 3 and 6 and a L-Asp in position 4 (Figure 1). Amino acid residues in position 2, 4 and 7 belong to the aliphatic group including Val, Leu and Ile (Peypoux et al. 1991; Itokawa et al. 1994; Bonmatin et al. 1995). A surfactin with Ala in position 4 was also observed (Peypoux et al. 1994). The presence of these variants can be related to alterations in the culture conditions, in particular, the feeding with some specific amino acid residues. This is a result from the mechanism of biosynthesis of such

Table 1 Peptide sequence and fatty acid chains of the different variants of each lipopeptide family from *Bacillus* spp.

Name	Primary structure of the peptide moiety	Main fatty acid chains	References
Fengycin family	Decapeptide with a lactone ring between carboxy-terminal group of Ile_{10} and OH group of Tyr_3	β-OH fatty acids	
Fengycin A	L-Glu-D-Orn-**D-Tyr**-D-a Thr-L-Glu-**D-Ala**-L-Pro-L-Gln-**L-Tyr**-L-Ile	aC_{15}, iC_{16}, nC_{16}	Schneider et al. 1999
Fengycin B	L-Glu-D-Orn-**D-Tyr**-D-a Thr-L-Glu-**D-Val**-L-Pro-L-Gln-**L-Tyr**-L-Ile	aC_{15}, iC_{16}, nC_{16}, C_{17}	Schneider et al. 1999
Plipastatin A	L-Glu-D-Orn-**D-Tyr**-D-a Thr-L-Glu-**D-Ala**-L-Pro-L-Gln-D-**Tyr**-L-Ile	nC_{16}, $_aC_{17}$	Nishikiori et al. 1986
Plipastatin B	L-Glu-D-Orn-**D-Tyr**-D-a Thr-L-Glu-**D-Val**-L-Pro-L-Gln-**L-Tyr**-L-Ile	nC_{16}, $_aC_{17}$	Nishikiori et al. 1986
Iturin family	Heptapeptide closed by a lactam ring with the β-NH_2 group of the acid chain	β-NH_2 fatty acids	
Bacillomycin D	**L-Asn**-D-Tyr-D-Asn-**L-Pro-L-Glu-D-Ser-L-Thr**	nC_{14}, iC_{15}, aC_{15}	Peypoux et al. 1981
Bacillomycin F	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Thr**	nC_{16}, iC_{17}, aC_{17}	Peypoux et al. 1985
Bacillomycin L or Lc[a]	**L-Asn**-D-Tyr-D-Asn-**L-Ser-L-Glu-D-Ser-L-Thr**	nC_{14}, iC_{15}, aC_{15}	Volpon et al. 2007
Iturin A	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	nC_{14}, iC_{15}, aC_{15}	Peypoux 1978
Iturin A_L	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	nC_{16}, iC_{16}	Winkelmann et al. 1983
Iturin C	**L-Asp**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	nC_{14}, iC_{15}, aC_{15}	Peypoux et al. 1986
Mycosubtilin	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Ser-L-Asn**	nC_{16}, iC_{16}, aC_{17}	Peypoux et al. 1986
Kurstakin family	Heptapeptide with a lactone ring between carboxy-terminal group of Gln_7 and OH group of Ser_4	β-OH fatty acid chain	
Kurstakin[b]	D-Thr-Gly-D-Ala-Ser-His-D-Gln-Gln	iC_{11}, nC_{12}, iC_{12}, iC_{13}	Hathout et al. 2000
Surfactin family	Heptapeptide closed by a lactone ring with the β-OH group of the fatty acid chain	β-OH fatty acids	
Bamylocin A[c]	**Glu-Leu-Met-Leu-Pro-Leu**-Leu-**Leu**	C_{13}	Lee et al. 2007
Esperin[d]	**L-Glu-L-Leu-D-Leu-L-Val-L-Asp**-D-Leu-L-**XE_7-COOH**	C_{13}, C_{14}, C_{15}	Thomas and Ito 1969
Lichenysin[e]	**L-XL_1-L-XL_2-D-Leu-L-XL_4-L-Asp**-D-Leu-L-**XL_7**	iC_{13}, aC_{13}, nC_{14} iC_{15}, aC_{15}	Lin et al. 1994
Pumilacidin	**L-Glu-L-Leu-D-Leu-L-Leu-L-Asp**-D-Leu-L-**XP_7**	aC_{15}, iC_{15}, nC_{16}, iC_{16}, aC_{17}, iC_{17}	Naruse et al. 1990
Surfactin	**L-Glu-L-XS_2-D-Leu-L-XS_4-L-Asp**-D-Leu-L-**XS_7**	iC_{14}, nC_{14} iC_{15}, aC_{15}	Peypoux et al. 1999

[a]Or bacillopeptin

[b]D forms of amino acid residue are deduced from NRPS modular structure (Bumpus et al. 2009; Abderrahmani et al. 2010)

[c]L and D forms are not specified

[d]The β-carboxyl of Asp_5 is engaged in the lactone

[e]Or halobacillin With XE_7 = Leu or Val, XL_1 = Gln or Glu; XL_2 = Leu or Ile; XL_4 and XL_7 = Val or Ile; XP_7 = Val or Ile; XS_2 = Val, Leu or Ile; XS_4 = Ala, Val, Leu or Ile; XS_7 = Val, Leu or Ile

compounds that involve non-ribosomal peptide synthetases (NRPS) (see Sect. 3). In such megaenzymes, the amino acid residue, which will be incorporated into the peptide, is first specifically recognised and activated by an adenylation domain. It was shown in several NRPS that the adenylation domains are not always specific and can accept some amino acid residues with similar structure. Interestingly, the presence of D-Leu seems specific, which could result from a higher specificity of the involved adenylation domain or of the epimerisation domains responsible for the transformation of the L-Leu to D-Leu. In position 1, two different amino acids are observed, Glu and Gln. It is stated in literature that lipopeptide with a Glu in position 1 are named surfactin, while those with a Gln are named lichenysin as they were discovered in the supernatant of *Bacillus licheniformis* culture (Horowitz et al. 1990). The presence of an Asn in position 4 was first mentioned in the structure described for lichenysin A by Yakimov et al. (1995) but the use of fast atom bombardment mass spectrometry allowed the same authors to definitively confirm the presence of an Asp as in surfactin (Yakimov et al. 1999). A surfactin-like compound was isolated from *Bacillus pumilus* culture supernatants and was called pumilacidin (Morikawa et al. 1992). Structural analysis shows that they correspond to Leu_4, Val_7 or Ile_7 surfactin. Esperin differs from the surfactin by a lactone ring involving the β-carboxyl of Asp in position 4 instead of the α-carboxyl of the terminal Leu. The β-hydroxy fatty acid chain linked to these different peptide moieties can contain 12–16 C atoms and show *n*, *iso* and *anteiso* configurations. The main fatty acid chains are usually C14 and C15. Recently a new lipopeptide, bamylocin A, was isolated from *Bacillus amyloliquefaciens* (Lee et al. 2007). The peptide chain of this molecule is Glu-Leu-Met-Leu-Pro-Leu-Leu-Leu. The molecular weight of the C13 form differs by less than 0.1 mass unit from the standard surfactin C14 isoform. This new result demonstrates the need of a precise MS-MS analysis of surfactin products to confirm their primary structure.

Surfactin-*O*-methyl ester was isolated from purified culture broth of different strains. Originally, it was considered to be the result of chemical modification, which occurs during the extraction procedure by methanol. The biological origin of such a methyl ester has been, however, confirmed by Liu et al. (2009) by using acetonitrile instead of methanol during the purification procedure of surfactin compounds produced by *Bacillus subtilis* HSO121.

2.2 The Family of Iturins

Iturin A, which is the main studied lipopeptide of the iturin family, is a heptapeptide interlinked with a β-amino acid fatty acid with a length from C14 to C17 (Peypoux 1978). Six other members of the iturin family were then described: iturin C, bacillomycin D, F, L and Lc and mycosubtilin (Bonmatin et al. 2003). All iturins have the same LDDLLDL chiral sequence with a common part of the peptide cycle: β-amino acid – L-Asx – D-Tyr – D-Asn. Except for Iturin C, the first amino acid of the peptide chain is L-Asn. Recent work from Volpon et al. (2007) confirms the presence

of L-Asn in position 1 of bacillomycin L instead of L-Asp initially described and thus demonstrates bacillomycin Lc and bacillomycin L to display the same structure. Contrary to most of the different variants of surfactin, which are synthesised by the same enzymes accepting different substrates, these different members of the iturin family probably results from different synthetases (see Sect. 3). In addition, the main length of the fatty acid chain differs from one member to another. Iturin A and C and bacillomycin D, L, each display a fatty acyl chain with a length of 14 and 15 C, while the C16 and C17 forms are the main representative fatty acid of the bacillomycin F and the mycosubtilin. A form of Iturin A with long fatty acyl chain (C16) was identified by Winkelmann et al. (1983) and called Iturin A_L.

2.3 Fengycin or Plipastatin, Who's Who?

This family of lipopeptides includes fengycins A and B, which are also called plipastatins. These molecules are lipodecapeptides, which differ by their amino acid residue in position 6 that can be Ala (form A) or Val (form B). They display an internal lactone ring in the peptidic moiety between the carboxyl terminal amino acid (Ile) and the hydroxyl group in the side chain of the tyrosine residue in position 3. Different β-hydroxy fatty acid chains (C14 to C18) are linked with an amide bond to the N-terminal amino acid residue (Glu) (Nishikiori et al. 1986; Vanittanakom et al. 1986). The main representative fatty acid chains are C15, C16 and C17. They are saturated, except in the case of a single lipopeptide isolated from the supernatant of *Bacillus thuringiensis*, which has a fatty acid chain with one double bond between carbons 13 and 14 (Kim et al. 2004). Two differences were initially identified between fengycin and plipastatin: a Gln instead of a Glu in position 8 and the L and D forms of tyrosine, which are in position 3 and 9, respectively, for plipastatins and 9 and 3 for fengycins. In the different works describing fengycin structure since its discovery, the presence of a Glu in position 8 was never mentioned. However, we confirmed the existence of fengycin molecule with a D-Tyr in position 3 from the supernatant of *Bacillus subtilis* S499 (Schneider et al. 1999). This result cannot be correlated with the structure of the synthetases described in other fengycin- or plipastatin-producing strains (see Sect. 3) (Tosato et al. 1997; Steller et al. 1999; Koumoutsi et al. 2004).

2.4 Other Lipopeptide Compounds, the Kurstakins

Kurstakins are a novel class of lipopeptides composed of several lipoheptapeptide with the same amino acid sequence: Thr-Gly-Ala-Ser-His-Gln-Gln (Hathout et al. 2000). Different fatty acyl chains (*iso*C11, *n*C12, *iso*C12 and *iso*C13) are linked via an amide bond to the N-terminal amino acid residue. Each lipopeptide has a lactone linkage between the carboxyl terminal amino acid and the hydroxyl group on the side chain of the serine residue. The L and D forms or the amino acid residues are

not yet characterised. However, the recent identification of the genes involved in the biosynthesis of such or similar compounds indicate that amino acids in 1 and 6 positions could be in the D-form (Bumpus et al. 2009; Abderrahmani et al. 2010) (see Sect. 3.5).

2.5 Conclusion

Each family of lipopeptides is constituted of several variants, which can differ in their fatty acid chain and their peptide moiety. The resulting wide diversity of molecules can be used to study the relationships between the structure and function of the lipopeptides. However, the existence of several different compounds with the same molecular weight shows that it is essential to precisely characterise their structure by using, for example, LC-MS-MS techniques.

3 Catalytic Assembly Lines for the Biosynthesis of Lipopeptides: From the Genes to the Biomolecules

In this section, we briefly discuss an overview of the NRPS, which is responsible for the biosynthesis of lipopeptides from *Bacillus* spp. We will then focus on the precise organisation of the catalytic assembly lines involved in the biosynthesis of surfactin and lichenysin and especially the spatial arrangement of the termination module of surfactin synthetase, a recently determined structure. A precise description of the other NRPS, which catalyse iturin A, mycosubtilin, bacillomycin D, fengycin, plipastatin and kurstakin, will be then given. Figure 2 summarises the modular organisation of all these multifunctional proteins.

3.1 Modular Enzymes: A Complex Catalytic Machinery Dedicated to the Biosynthesis of Secondary Metabolites

Here, we describe the enzymatic machinery for the non-ribosomal synthesis of peptides.

3.1.1 Discovery of the Non-ribosomal Peptide Synthesis

In 1968, Gevers et al. demonstrated for the first time using cell extracts of the producer strains that biosynthesis of gramicidin (a peptide antibiotic) is possible in the presence of RNases or inhibitors of the ribosomal machinery. It was

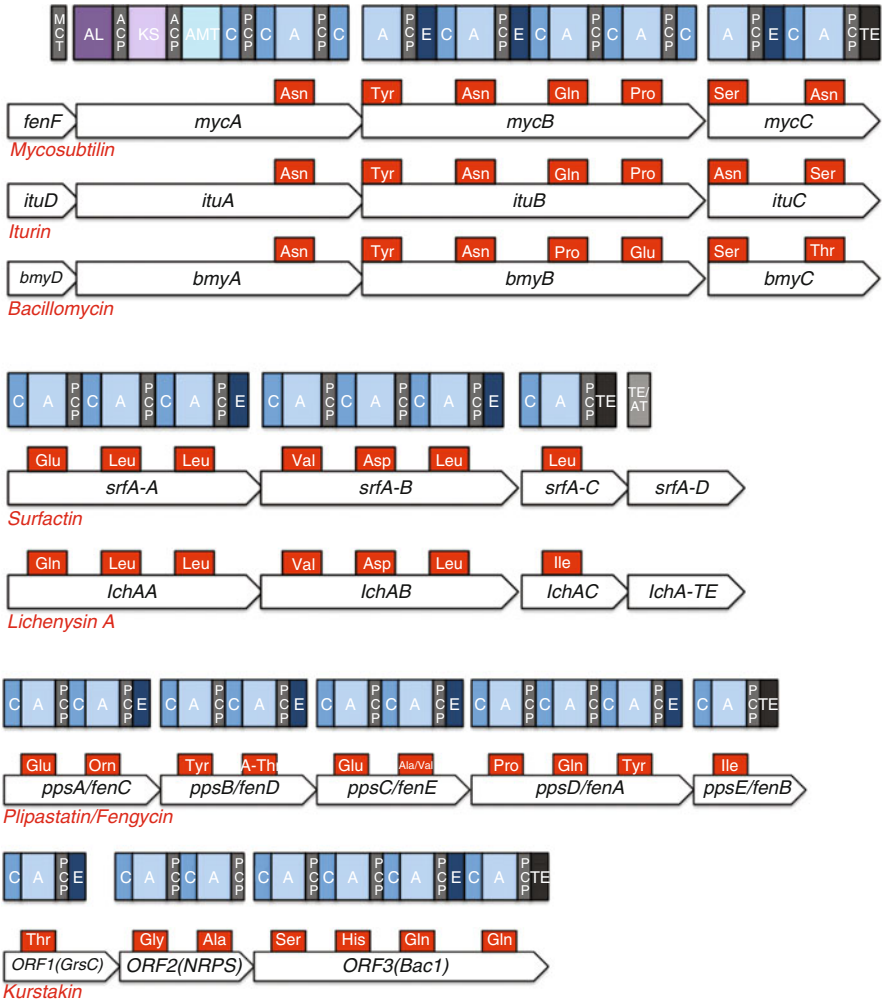

Fig. 2 Operons responsible for lipopeptide biosynthesis in *Bacillus* spp. Domains are described in the text

a key experiment to show that another biosynthetic pathway exists for the biosynthesis of the peptides. Since this discovery, many works were carried out to describe in detail the non-ribosomal peptide biosynthesis (Finking and Marahiel 2004). It is responsible for the synthesis of more than 1,000 active biomolecules that can be gathered in about 200 families. A recent database (NORINE) compiles most of the non-ribosomal peptides (NRPs) (Caboche et al. 2008).

3.1.2 The Main Catalytic Domains

Non-ribosomal peptide synthesis involves large multienzymatic proteins called non-ribosomal peptide synthetases (NRPS), which are organised in modules (Sieber and Marahiel 2005). Each module is responsible for the incorporation of one building block into the growing polypeptide chain and can be subdivided in a defined section of the protein called a domain involved in a specific enzyme activity. Four main domains are present in most of the NRPS: adenylation (A), thiolation (T), condensation (C) and thioesterase (TE) domains. The adenylation domain selects the cognate amino acid and activates it as amino acyl adenylate. Several crystal structures of A-domains have been solved to date. The first two structures were the phenylalanine-activating A-domain of the gramicidin S-synthetase A (GrsA) from *Bacillus brevis* (Conti et al. 1997) and the 2,3-DHB-activating A-domain DhbE from *B. subtilis* (May et al. 2002). These crystal structures facilitate the assignment of ten amino acid residues that play a decisive role in the coordination of the substrate. The so-called non-ribosomal code can be used to predict an A-domain selectivity on the basis of its primary sequence (Stachelhaus et al. 1999). The activated amino acid is then transferred to the T-domain, also called peptidyl-carrier protein (PCP), as it is the transport unit of the activated intermediate. In such a domain, the activated amino acid residue is covalently tethered to its 4′phosphopantheteinic (4′-PP) cofactor as thioester. This cofactor is post-translationally transferred to a serine of the PCP. This reaction is catalysed by a phosphopantetheinyl transferase (encoded in *Bacillus subtilis* by the *sfp* gene), which is thus essential to transform apoform of NRPS in its holoform (Mofid et al. 2004). The 4′PP cofactor acts as a flexible arm to allow the bound amino acyl and peptidyl substrate to travel between different catalytic centres. The C-domain catalyses the formation of the peptide bond between amino acyl substrate bound to PCPs of adjacent modules. The termination of synthesis is catalysed by the terminal enzyme of the last module. In most cases and for lipopeptide synthesis, this reaction is performed by a thioesterase domain (TE). This allows the release of the peptide and is also frequently involved in the formation of a macrocyclic product (lactones and lactams) or the oligomerisation of peptide units (Kopp and Marahiel 2007). Other alternative release mechanisms can be achieved by the reduction of the peptidyl-S-PCP final product to generate a linear aldehyde or alcohol.

3.1.3 Secondary Catalytic Domains

Additional enzymes can be involved in the biosynthesis of the peptide to modify the structure of the monomer involved in the primary structure or to add some external compounds to the peptide. Some of these are integral parts of the NRPS and act in *cis* whereas others are distinct enzymes acting in *trans* before full maturation of the NRPS product. Among these tailoring domains, which include cyclisation (Cy), methylation (Me), oxidation (Ox), glycosylation, epimerisation (E) and addition of fatty acid chain, the final two are involved in lipopeptide biosynthesis in *Bacillus*

spp. The E-domain catalyses the epimerisation of the PCP-bound L-amino acid of the growing polypeptide chain. The addition of the fatty acid chain to the first amino acid of the peptide moiety is catalysed by a first specific condensation domain, also called starter condensation domain. The added fatty acid chain can itself be partially synthesised by another main group of modular enzymes, the polyketide synthases (PKS). In this last case, a hybrid PKS/NRPS is required for the synthesis of the biomolecules (Du et al. 2001).

3.1.4 Protein–Protein Interactions

In most multienzyme complexes, several proteins are involved in the complete assembly lines. The biosynthesis of the right NRP requires the proper protein–protein interaction between partner enzymes and concomitantly prevents undesired interactions between non-partner enzymes. Short terminal structures, referred to as NRPS communication-mediating (COM) domains are responsible, at least for the most part, for the correct channelling of reaction intermediates along the assembly line (Hahn and Stachelhaus 2004). A donor COM domain COMD X, situated at the C terminus of an aminoacyl- or peptidyl-donating NRPS "X", and an acceptor COM domain COMA Y, located at the N terminus of the accepting partner enzyme "Y", form a compatible (cognate) pair that is crucial for establishing the productive interaction between both enzymes. In contrast, within a hypothetical assembly line "X Y-Z", the COM domains COMD X and COMA Z of the non-partner NRPSs "X" and "Z" are considered incompatible (non-cognate), preventing their futile contact. Accordingly, the establishment of a defined assembly line and synthesis of a distinct NRP product is ensured by the grouping of exclusively cognate pairs of COM domains. Donor and acceptor NRPS COM domains comprise 15–30 amino acid residues.

3.2 Non-ribosomal Peptide Synthesis of Surfactin and Lichenysin

The structure of the enzymatic complexes participating in the synthesis of surfactin and lichenysin has been described in detail and is presented in this section.

3.2.1 The Surfactin Operon

Three large Open Reading Frames (ORFs) coding for surfactin synthetases are designated *srfA-A*, *srfA-B* and *srfA-C* (Galli et al. 1994). They present a linear array of seven modules (one module per residue), three modules are present in the products of SrfA-A and SrfA-B, respectively, and the last one in SrfA-C. A high specificity was observed for adenylation domains of modules 1, 3, 5 and 6, which recognised L-Glu, L-Leu, L-Asp and L-Leu amino acid residues, respectively. Modules 3 and 6

contain an epimerase domain (E), which transforms the incorporated L-Leu in a D-Leu. In vitro studies of the specificity of adenylation domains of modules 2, 4 and 7 show that they are able to accept several aliphatic amino acid residues as substrates. The β-hydroxylated fatty acid chain is added to the amino acid activated in the first module by the way of a starter condensation domain. A first thioesterase fused with the carboxy-terminal end of the final activation PCP domain is responsible for the release of the synthesised product from the enzymatic template. This enzyme also catalyses the lactone bond formation between the carboxylate group of the last amino acid and the hydroxyl group of the fatty acid chain. Chemoenzymatic approaches were used to characterise the specificity of the thioesterase domain. Different heptapeptides were synthesised by chemical means, and a thioesterase domain was cloned and produced as an isolated enzyme. The presence of Glu in position 1 and the two Leu in positions 6 and 7 are essential for substrate recognition by the thioesterase domain. A second thioesterase/acyltransferase (TE/At-domain) encoded by a fourth gene, *srfA-D*, stimulates the initiation of the biosynthesis. Two cognate-pairs of communication-mediating (COM) domains, $COM^{D}_{SrfA\text{-}A}$/$COM^{A}_{SrfA\text{-}B}$; $COM^{D}_{SrfA\text{-}B}$/$COM^{A}_{SrfA\text{-}C}$, facilitate the selective interaction between partner enzymes (Chiocchini et al. 2006).

A small gene, designated *comS*, is located within the coding region of the fourth amino acid-activation domain of *srfA* and thus co-expressed with the *srfA* operon. This gene is required for competence development in *Bacillus subtilis* but not directly involved in the biosynthesis of surfactin (Hamoen et al. 1995).

3.2.2 The Lichenysin Operon

The same organisation in three ORFs was described for lichenysin operon (Konz et al. 1999). Only small differences were observed in the primary sequences of the corresponding synthetases, especially in the ten amino acids representing the Non-ribosomal code, present in the active site of adenylation domains of module 1 and 7. In the module 1, the modification of a Lys to Glu led to the incorporation of a Gln instead of a Glu in position 1 of the lipopeptide. In the module 7, the replacement of an Ala by a Gly and a Cys by a Val in the active site of the adenylation domain favours the incorporation in the peptide formed of an Ile instead of a Leu.

3.2.3 Structure of an Entire Termination Module

A variant of the termination module SrfA-C of the surfactin synthetase, in which the active site serine of PCP was changed into alanine by site-directed mutagenesis, provided diffracting crystals (Tanovic et al. 2008). The structure of the SrfA-C variant was solved at 2.6 resolution and covered the entire module with its four catalytic domains and linker regions in between. This is the first example of the resolution of the complete structure of a NRPS module. The SrfA-C structure provided unique information on how the essential catalytic

domains are orientated and spatially arranged. Overall, the structural core of the module is a compact rectangular catalytic platform mainly built by the intimate association of the C-domain and the large N-terminal part of the A-domain. Both active sites of the A- and C-domains are arrayed on the same side of the platform. The C-terminal lid region of the A-domain (~100 residues of the C-terminal part) and the PCP domains are tethered to each other on top of the platform and connected to the large N-terminal region of A by a flexible linker of 15 residues. In this arrangement, PCP and A-lid region can easily move relative to the static C–A-platform. In contrast to the compact C–A-PCP domain association, the terminal TE domain builds an independent fold connected through a short (9 residues) linker to PCP and essentially shows an independent α/β-fold, identical to that of the dissected TE-domains (Marahiel 2009).

3.3 The Hybrid PKS/NRPS Complex Involved in Iturin Biosynthesis

Contrary to surfactin and fengycin, iturin derivatives are synthesised by a PKS–NRPS hybrid complex. The operon consists of four ORFs called *fenF*, *mycA*, *mycB* and *mycC* for mycosubtilin (Duitman et al. 1999), *ituD*, *ituA*, *ituB* and *ituC* for iturin (Tsuge et al. 2001a) and *bmyD*, *bmyA*, *bmyB* and *bmyC* for bacillomycin D (Hofemeister et al. 2004; Koumoutsi et al. 2004). The last three genes encode for the three NRPSs, which are responsible for the incorporation of the first residue (Asn for MycA, ItuA and BmyA), the following four residues (Tyr, Asn, Gln, Pro for MycB and ItuB; Tyr, Asn, Pro, Glu for BmyB) and the two last residues (Ser, Asn for MycC; Asn, Ser for ItuC and Ser, Thr for BmyC). The thioesterase present in the last module catalyses the release of the peptide and the formation of an amide bond between the carboxylic group of the last amino acid and the amino group of the fatty acid chain. Epimerisation domains were identified in modules 2, 3 and 6. D-form of the corresponding amino acid residues is observed in the final product. The difference between structures of iturin A and mycosubtilin in which the last amino acids are inverted can be explained by an intragenic domain exchange between *mycC* and *ituC*. The synthetase of bacillomycin D is similar to that of iturin A except for the amino acid residues activated by modules 4, 5 and 7, which are Pro, Glu and Thr, respectively. *FenF* (*ituD*) encodes a malonyl-CoA transacylase (MCT-domain), and the *mycA* also contains genes related to PKS. These genes are responsible for the last steps of the biosynthesis of the fatty acid chain [last elongation and β-amination (Aron et al. 2005)] before its transfer to the first amino acid of the peptidic moiety [acyl-CoA ligase (AL-domain), acyl carrier protein (ACP-domain), β-keto acyl synthetase (KS-domain) and amino transferase (AMT domain)]. Hansen et al. (2007) have shown that the AL-domain is able to activate

free fatty acids through an acyl-adenylate intermediate and loaded on the adjacent thiolation domain independently of co-enzyme A.

3.4 Non-ribosomal Peptide Synthesis of Fengycin and Plipastatin

Fengycin or plipastatin are synthesised by five NRPSs (Fen1 to Fen5) encoded by an operon with five ORFs *fenA-E* (or *ppsA-E*) (Tosato et al. 1997; Steller et al. 1999; Koumoutsi et al. 2004). The first three enzymes, Fen1, Fen2 and Fen3 contain two modules, the fourth contains three modules and the last enzyme consists of one module. Fen1 activates and incorporates glutamate in position 1 and ornithine in position 2, which form the side chain of the peptidic moiety of fengycin. Fen2 is responsible for the activation and incorporation of tyrosine in position 3 and allo-threonine in position 4. Fen3 activates and incorporates glutamate in position 5 and alanine or valine in position 6. Fen4 is a three modular enzyme, which catalyses the activation and incorporation of proline in position 7, glutamine in position 8 and tyrosine in position 9, and Fen5 allows the incorporation of the last amino acid residue: isoleucine in position 10. Like in surfactin biosynthesis, the β-hydroxylated fatty acid chain is added to the amino acid activated in the first module by the way of a starter condensation domain. The thioesterase present in the last module catalyses the release of the peptide and the formation of an ester bond between the carboxylic group of the last amino acid (Ile) and the hydroxyl group of a tyrosine in position 3. Epimerisation domains were identified in modules 2, 4, 6 and 9. D-form of the corresponding amino acid residues is observed in the final product. This depicts the structure of the plispastatin; however, it does not correlate with fengycin produced by *Bacillus subtilis* S499. In the latter case, a D-Tyr is present in position 3 of the peptide and an L-Tyr in position 9 (Schneider et al. 1999). However, the organisation of the fengycin operon, which was not yet described for this strain could be different.

3.5 The Recent Discovery of the Biosynthesis Mechanism of Kurstakin

Two new approaches were recently developed to detect new NRPS genes responsible for the biosynthesis of lipopeptide compounds. The first being a PCR approach using degenerated primers based on the intraoperon alignment of adenylation and thiolation nucleic acid domains of all enzymes implicated in the biosynthesis of each lipopeptide family (Tapi et al. 2010). For the second approach (Bumpus et al. 2009), the authors took advantage of the size of the NRPS enzymes and the presence of unique marker ions derived from the common phosphopantetheinyl cofactor to adapt mass spectrometry-based proteomics to selectively detect NRPS and PKS gene clusters in microbial proteomes without requiring genome sequence

information. In both cases, authors discovered in *Bacillus thuringiensis* (Abderrahmani et al. 2010) and *Bacillus cereus* the genes involved in the biosynthesis of the kurstakin. The exponential growth of genome sequences and the development of such interesting tools to detect or predict potentially novel NRPs highlight the interest of a database, such as NORINE, which collects all the known structures of NRPs and provides software for efficient structure comparison (Caboche et al. 2009). A good example is the pseudo-discovery of bacillorin, the synthetase sequences of which have been deposited in GenBank since 2008. The peptide was assumed to be a novel compound synthesised by NRPS. However, it was demonstrated to be the bacillomycin L, based on predictive amino acid incorporation followed by structural pattern comparison with all peptides annotated in the NORINE database (Leclère, personal communication). Bacillorin and bacillomycin L should then be considered as synonymous names for a single molecule.

3.6 Conclusion

Up to now, all the lipopeptides produced by *Bacillus* spp. are synthesised by NRPS. These modular enzymes have been especially well studied in the case of surfactin. These "megaenzymes" exhibit a broad spectrum of activities, conferring them an extraordinary potential for the development of bioprocesses, which lead to the biosynthesis of novel compounds for pharmaceutical, agricultural and biotechnological sectors.

4 A Complex Regulation of the Biosynthesis

In this section, the molecular mechanism of regulation of the expression of the operon involved in the biosynthesis of surfactin and other lipopeptides from *Bacillus* are first described. Figure 3 shows the cascade of regulation, which controls surfactin operon expression. The environmental factors, which mainly influence the production of the lipopeptides, are also discussed.

4.1 Quorum Sensing and Surfactin Efflux

Surfactin production is quorum sensing-dependent. Its regulation involves several pheromones ComX, PhrC, PhrF, PhrG and PhrH together with several pleiotropic regulators such as CodY, DegU and AbrB. These form a complex cascade governing multiple differentiation pathways, such as sporulation and competence (Hamoen et al. 2003; Hayashi et al. 2006). Cosby et al. (1998) also showed that surfactin synthetase expression is pH dependent. A surfactin-susceptible mutant

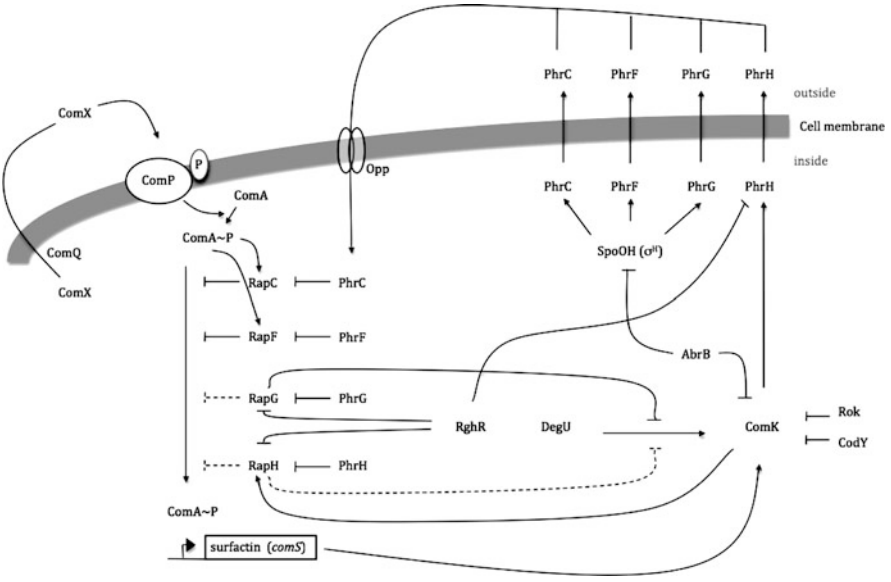

Fig. 3 Regulatory cascade of surfactin operon expression. Bacteria are able to communicate with each other through the production of "quorum sensing" pheromones. Accumulation of these pheromones in the growth medium signals the presence of a sufficient number of congeners (a quorum) and triggers various cell density-dependent processes. Expression of the surfactin operon is one of these processes. Two types of pheromones are involved in the regulation of *srfA* operon. The first pheromone is ComX, which is secreted with the help of ComQ in the culture medium. ComP senses the accumulation of the ComX, and at critical ComX concentration, phosphorylates itself. Subsequently, autophosphorylated ComP phosphorylates ComA. ComA–P activates expression of surfactin operon and ComS, which is an ORF encoded within de *srfA* mRNA. ComS leads to the autoactivation of ComK. In addition, several activators such as ComK itself and DegU and repressors including CodY, AbrB and Rok are involved in ComK activation. The second type of pheromones is the group of Phr peptides: PhrC, PhrF, PhrG and PhrH. They are probably first synthesised as small proteins, which are secreted, processed in pentapeptides and then internalised by an oligopeptide permease (Opp). The *phrH* gene constitutes an operon with a *rapH* gene. Both genes are thus activated by ComK. Products of *phr* and *rap* genes are interacting regulator factors that modulate the phosphorylation state or DNA-specific response regulators. The *phrC, phrF* and *phrH* genes need the σ^H (Spo0H) form of RNA polymerase to be transcribed. RapC, RapF and, in certain circumstances, RapG and RapH, are inhibitors of the DNA binding of ComA–P. ComA–P activates *rapC* and r*apF*. PhrC, PhrF, PhrG and PhrH inhibit their cognate Rap proteins. RapG and RapH inhibit DNA binding of DegU. RghR is a r*apG*, *rapH* and *phrH* repressor

was obtained by transposon mutagenesis. Genetic analysis revealed *yerP*, a gene that would be involved in surfactin self resistance. It has homology with resistance, nodulation and cell division (RND) family proton motive force-dependent efflux pumps. It should be responsible for surfactin efflux. Its expression is highest at the end of logarithmic phase (Tsuge et al. 2001b). Duitman et al. (2007) have shown recently that mycosubtilin synthetase expression is under the influence of a regulatory cascade depending on AbrB, one of the main transition state regulators in *B.*

subtilis. In contrast to surfactin, the expression of mycosubtilin synthetase is lowest in minimal medium and highest in rich medium. We recently demonstrated that mycosubtilin synthetase expression is oxygen-dependent (Guez et al. 2008). To our knowledge, little information is available about the regulation mechanism of fengycin or plipastatin biosynthesis. However, the introduction of a pleiotropic regulator DegQ in *Bacillus subtilis* 168 results in a tenfold increase in the production of plipastatin (Tsuge et al. 1999).

4.2 Influence of Environmental Factors

A lot of studies have pointed out different environmental factors for their effect on surfactin production (Peypoux et al. 1999; Akpa et al. 2001). Several experiments performed in our laboratory have shown that this effect can be strain dependent. Regarding the carbon source, glucose was the most used substrate. Saccharose and fructose have also been mentioned as efficient carbon sources contrary to glycerol and hexadecane. The two main culture media mentioned in literature for surfactin production are Cooper's medium, which contain mineral nitrogen source (NH_4NO_3) (Cooper et al. 1981), and Landy's medium (Landy et al. 1948), with glutamic acid as a nitrogen source. Studies of the mineral requirement clearly established the need and the stimulatory effect of iron and manganese (Wei et al. 2004). For continuous operation, a critical nitrogen/iron/manganese molar ratio of 920:7.7:1.0 was determined and was found to sustain surfactin production for at least 36 generations (Sheppard and Cooper 1991). Oxygen and temperature are also considered as important parameters. Higher temperatures (37°C) favoured surfactin synthesis of *Bacillus subtilis* RB14 isolated from compost (Ohno et al. 1995a) and ATCC6633 but not of *Bacillus subtilis* S499 (Jacques et al. 1999). The replacement of Cooper's nitrogen source and the introduction of oxygen limitation, which redirects the energy flux into product synthesis, have led to the highest productivity mentioned for surfactin production (7 g l^{-1}) by *Bacillus subtilis* C9 (Kim et al. 1997). Such a process appears not to be adapted to *Bacillus subtilis* S499, which produced higher surfactin yield in better aeration conditions (Hbid et al. 1996; Jacques et al. 1999).

Production of lichenysin by *Bacillus licheniformis* was conducted in anaerobic conditions and mainly at higher temperatures (up to 45°C) than for surfactin. Presence of salt (NaCl 5%) was also sometimes required (Yakimov et al. 1995). However, production yields were in average, five to ten times lower than with surfactin (Yakimov et al. 2000).

A 30-fold increase in mycosubtilin production was observed when the temperature was decreased from 37 to 25°C. This was observed both for strain ATCC6633 and its derivative BBG100, which is a constitutive mycosubtilin over-producer. However, no significant difference in either the expression of the mycosubtilin synthetase encoding genes or in the intracellular synthetase concentration could be

found, suggesting that the observed phenotype originated from a higher mycosubtilin synthetase turnover at lower temperature (Fickers et al. 2008).

Recently, our laboratory demonstrated that environmental conditions can drastically modify the ratio between surfactin and fengycin produced by a derivative strain of *Bacillus subtilis* ATCC21332. Presence of polypropylene carriers in a batch culture (Gancel et al. 2009) or use of a polypropylene membrane (Coutte et al. 2010a) to aerate bioreactors (bubbleless process see Sect. 6) increases the biosynthesis of fengycin.

4.3 Conclusion

Many of the physico-chemical parameters influence the biosynthesis of lipopeptides from *Bacillus* spp. This is certainly due to the complex regulation of lipopeptide operon expression involving several pleiotropic regulators. However, intracellular pools of synthetase substrates (amino acid residues and fatty chain) and turn-over of the enzymes have also to be taken into account. To our knowledge, a limitation of lipopeptide secretion was never described in literature.

5 Physico-chemical Properties and Biological Activities: A Strong Relationship

The content of this section is a summary of the main physico-chemical properties of surfactin, iturin and fengycin compounds, biological activities and their possible relationships. The mechanisms of action, where known, are also described. To end with, the multifunctional role of lipopeptide in plant protection against phytopathogen is also discussed.

5.1 Surfactin: A Potent Biosurfactant, Which Combines High Effect on Surface Tension and Low Critical Micellar Concentration

Surfactins are powerful biosurfactants with exceptional emulsifying and foaming properties (Razafindralambo et al. 1996). They are able to reduce surface tension of water to 27 mN m^{-1} and show a low critical micellar concentration (CMC) of about 10 mg l^{-1}. Due to their amphiphilic nature, surfactins can also readily associate and tightly anchor into lipid layers. It can thus interfere with biological membrane integrity in a dose-dependent manner. The effect of surfactin on artificial membrane was studied in detail on different models using atomic force microscopy (Deleu et al. 1999a, 2001;

Francius et al. 2008), light scattering, differential scanning calorimetry, small-angle neutron scattering and cryo-electron microscopy (Kell et al. 2007). Studies on lipid vesicles suggest that at low concentration (surfactin-to-lipid mole ratio R_b lower than 0.04 in the membrane), surfactins insert exclusively in the outer leaflet of the membrane inducing only limited perturbation. At intermediate concentration (R_b 0.05–0.1), it provokes a transient permeabilisation, but membranes re-anneal. Irreversible pore formation then occurs at higher ratio (R_b 0.1–0.2) due to the insertion of surfactin-rich clusters in the membrane. Further addition of surfactins to reach the CMC leads to complete disruption and solubilisation of the lipid bilayer with formation of mixed micelles (R_b 0.22) (Heerklotz and Seelig 2007; Carrillo et al. 2003). Interestingly, the presence of cholesterol in the phospholipid layer attenuates the destabilising effect of surfactins, which suggests that the susceptibility of biological membranes may vary in a specific manner, depending on the sterol content of the target organisms. This could explain why surfactins display haemolytic, antiviral (Kracht et al. 1999), antimycoplasma (Vollenbroich et al. 1997) and antibacterial activities but, intriguingly, no marked fungitoxicity. However, recent studies have shown that surfactin could induce plant systemic resistance (Ongena et al. 2007; Jourdan et al. 2009). The molecular mechanism of this induction is not yet known but could also involve interaction with the membrane of plant cells. The lipid bilayer destabilisation process, observed with membrane models, is facilitated by the tri-dimensional form of the surfactin molecule. In solution, the peptide moiety of surfactin shows a "horse-saddle" topology. Its two negatively charged amino acid residues (Asp and Glu) form a claw and function as binding sites for mono- and divalent cations, while its fatty acid chain is extending at the opposite side of the ring. In membranes, charged side chains are protruding into the aqueous phase and apolar moieties reaching into the hydrophobic core of the membrane (Deleu et al. 2003). The use of chemically modified or biosynthetic variants has revealed prominent roles for some sub-structures (Morikawa et al. 2000). The esterification of Glu residue in surfactin increases its surface active power. The association properties of the lipopeptide increase with the diminution of their anionic charges. Surfactin mono methyl-ester has higher haemolytic activity than surfactin. But linear surfactin is less haemolytic (Dufour et al. 2005).

The emulsification properties of surfactin were also used to solubilise xenobiotic compounds (Lai et al. 2009) and enhance their biological degradation (Whang et al. 2009) or to facilitate oil recovery from carbonate reservoirs (Zhang et al. 2000). When linear surfactin was prepared by saponification of the lactone ring, its oil displacement activities decreased to one-third of their respective original value (Morikawa et al. 2000).

Several studies have also shown the role played by surfactin in pellicle formation of the producing *Bacillus* strain at the air-water interface (Hofemeister et al. 2004; Chollet-Imbert et al. 2009), in swarming (Julkowska et al. 2005; Debois et al. 2008) or in biofilm formed on roots (Bais et al. 2004). At concentrations between 5 and 50 mg l^{-1}, surfactin inhibits *Salmonella* biofilm formation in microtiter plates and urethral catheter (Mireles et al. 2001),and *Pseudomonas syringae* growth on roots (Bais et al. 2004). The adsorbtion of surfactin on Stainless steel or Teflon substrata deeply modified the hydrophobicity of the surface and the *Bacillus cereus* spore attachment

(Shakerifard et al. 2009). Surfactin can also modify the surface hydrophobicity of the producing strain (Ahimou et al. 2000). Neutron reflectometry was recently used to study the structure of surfactin at the air-water and hydrophobic solid/water interfaces and on surfaces. Surfactin was found to adopt a ball-like structure with a thickness of $14 \pm {}_1$Å and an area per molecule of 147 ± 5 Å^2 (Shen et al. 2009). The structure of surfactin micelles has been examined by means of small-angle neutron scattering. The aggregation number was found to be unusually small at 20 ± 5.

Furthermore, anti-inflammatory (Kim et al. 1998), anticancer activity (Kameda et al. 1974) and immunomodulatory effects (Park and Kim 2009) were also determined for surfactin. Cao et al. (2009) showed that surfactin induces apoptosis and G2/M arrest in human breast cancer MCF-7 Cells.

5.2 *Iturin: A Strong Antifungal Compound*

Iturin is also a biosurfactant but less potent than surfactin (Deleu et al. 1999b). It reduces the surface tension of water to 43 mN m^{-1} and forms at CMC (about 20 mg l^{-1}) micelles with a Stokes radius of 1.3 nm and an aggregational number of 7. At concentrations slightly higher than CMC, iturin probably forms a fully interdigitated bilayer where each hydrocarbon tail spans the entire hydrocarbon width of the bilayer, resulting in multilamellar vesicles with an average size of 150 nm (Grau et al. 2001).

Though they are also strongly haemolytic, the biological activity of iturins differs from surfactins: they display a strong in vitro and in vivo antifungal action against a large variety of yeast and fungi but have only limited antibacterial and no antiviral activities (Leclère et al. 2005; Mizumoto et al. 2007; Romero et al. 2007; Fickers et al. 2009). This fungitoxicity of iturins almost certainly relies on their membrane permeabilisation properties. However, the underlying mechanism is based on osmotic perturbation due to the formation of ion-conducting pores and not membrane disruption or solubilisation as caused by surfactins (Aranda et al. 2005). In addition to the direct activity against fungi, iturin derivatives enhance the invasive growth of the producing strain, and thus, by these two mechanisms participate in plant protection against phytopathogens (Leclère et al. 2005, 2006).

The length of the fatty acid chain, the presence of an Asp in position 1 and tyrosine residue in position 2 are important structural traits for the antifungal activity of the lipopeptide (Bonmatin et al. 2003; Fickers et al. 2009).

5.3 *Fengycin and Plipastatin: Immunomodulators in Plants and Animals*

Fengycins are less haemolytic than iturins and surfactins but retain a strong fungitoxic activity more specifically against filamentous fungi. Mechanistically,

the action of fengycins is less known compared to other lipopeptides, but they also readily interact with lipid layers and somewhat retain the potential to alter cell membrane structure (packing) and permeability in a dose-dependent way. Immunosuppressive activity of plipastatin was also described and patented (Umezawa et al. 1988).

Lipopeptides are known to act in a synergistic manner as suggested by several studies, on surfactin with iturin (Maget-Dana et al. 1992; Razafindralambo et al. 1997), surfactin with fengycin (Ongena et al. 2007) and iturin with fengycin (Romero et al. 2007).

5.4 *Lipopeptides: Versatile Weapons for Biocontrol of Plant Diseases*

In the context of biocontrol of plant diseases, the three families of *Bacillus* lipopeptides (surfactins, iturins and fengycins) were at first mostly studied for their antagonistic activity against a wide range of potential phytopathogens including bacteria, fungi and oomycetes. Recent investigations have shed light on the fact that these lipopeptides can also influence the ecological fitness of the producing strain in terms of root colonisation and thereby persistence in the rhizosphere, also playing a key role in the beneficial interaction with plants by stimulating host defence mechanisms (Ongena et al. 2005, 2007). The different structural traits and physico-chemical properties of these effective surface- and membrane-active amphiphilic biomolecules explain their involvement in most of the mechanisms (Ongena and Jacques 2008).

5.5 *Conclusion*

The large panel of physico-chemical properties and biological activities of lipopeptides from *Bacillus* has to be correlated to their remarkable structure diversity. They offer them various potential applications. Their complementarity and the observation of some synergistic effects entice interest to further study the different families.

6 New Strategies for an Optimal Production of Novel or Existing Lipopeptidic Compounds

In this section, we first discuss the molecular strategies used to achieve higher yield of lipopeptide biosurfactants or to obtain modified compounds. Then we give a brief overview of the different techniques used to produce, purify and quantify the lipopeptide from *Bacillus subtilis*.

6.1 Surfactin Synthetases Re-engineering

Surfactin synthetase was the first NRPS for which a genetic re-engineering experiment was reported. It concerns the terminal module, which incorporates leucine in the natural system and exhibits the domain composition C-A-PCP-TE. The activating and covalent attachment domains (A-PCP) were exchanged by A-PCP units from bacterial and fungal origin with various amino acid specificities. Novel surfactin variants with aliphatic (Val), charged (Orn) and aromatic (Phe) residues at position 7 were created and their structure was confirmed by mass spectrometry (Stachelhaus et al. 1995). All these new variants displayed the same haemolytic activity as the native surfactin. However, low yields of the peptide products (0.1–0.5% in comparison to the parent strain) were observed probably due to the high selectivity of C domains in the acceptor site for the cognate amino acid substrate. Different results were obtained with deletion of the Leu incorporating module 2. Initially unsuccessful, a second trial yielded the predicted surfactin deprived of the second Leu residue in about 10% yield in comparison to the parent strain (Mootz et al. 2002). This highlighted the importance of precise linker surgery. The recombination of whole modules represents a rather drastic intervention in NRPS biosynthesis, which usually results in reduced catalytic efficiency and product yield. However, Yakimov et al. (2000) report the entire replacement of module 1 and 5 of surfactin synthetase by those of lichenysin synthetase, to create a fully active hybrid enzyme that forms lichenysin-like biosurfactant in high yields. A more conservative strategy involves manipulating the A domain's specificity through point mutations affecting the substrate-coordinating amino acid residues according to the "non-ribosomal code" (see A-domain section). For example, the substrate specificity of the Glu-activating module 1 of surfactin synthetase was rationally altered in this way. A single point mutation changed the specificity from Glu to Gln without a decrease in catalytic efficiency. A second mutation changing the specificity of module 5 from Asp to Asn yielded the expected surfactin derivative in vivo (Eppelmann et al. 2002).

6.2 Combinatorial Synthesis of Lipopeptides

Up until now, corresponding approaches were generally limited to module swaps, which: (1) require major manipulations of the biosynthetic template, (2) were mostly connected with significant decrease in product titer, and (3) constitutionally lead to the synthesis of only one product per experiment. Chiocchini et al. (2006) show that a heterologous COM domain pair can replace the native COM domain pair. Indeed, the replacement of $COM^{D}_{SrfA\text{-}A}/COM^{A}_{SrfA\text{-}B}$ by $COM^{D}_{TycB\text{-}A}/COM^{A}_{TycC}$; a cognate COM domain pair facilitating the interaction between the two NRPSs, TycB and TycC, involved in the biosynthesis of tyrocidine in *Bacillus brevis* only caused a minor decrease in surfactin production. Using different modifications of COM

domain pairs, the authors also show that it was possible to skip the srfA-B domain and to generate in majority a lipotetrapeptide instead of the natural lipoheptapeptide.

6.3 Directed Biosynthesis and Molecular Optimisation

Bacillus strains frequently co-produce different families of lipopeptides (Jacques et al. 1999). Several strategies were thus proposed to selectively overproduce a family or only a variant of a family. Firstly obtained by random mutagenesis (Lin et al. 1998; Yoneda et al. 2002), overproducing mutants were then constructed by promoter exchange. The four main promoters used are P_{repU} (Tsuge et al. 2001a; Leclère et al. 2005; Coutte et al. 2010a), P_{xyl} (Fickers et al. 2009), P_{spac} (Sun et al. 2009) and P_{amyQ} (Ongena et al. 2007). Disruption mutants were also obtained by introducing resistance genes into synthetase operons (Coutte et al. 2010a). Lin et al. (1998) isolated a *Bacillus licheniformis* mutant derived by random mutagenesis with *N*-methyl-N9-nitro-*N*-nitrosoguanidine, which produces 391 mg l^{-1} of lichenysin. Using the same approach, Yoneda et al. (2002) described in a patent, a production yield of up to 50 g l^{-1} of surfactin. The replacement of the native promoter of iturin (Tsuge et al. 2001a) and mycosutilin (Leclère et al. 2005) operons by P_{repU} led to an overproduction of respectively 3 and 15 times of the lipopeptide to reach a concentration of about 200 mg l^{-1} in both cases. Performed on surfactin synthetase, the same promoter exchange did not allow the improvement of the surfactin production (Coutte et al. 2010a). The native promoter P_{srf} of *Bacillus subtilis* 168 is considered very efficient, frequently allowing the concentration of surfactin in the culture supernatant to be higher than 1 g l^{-1} (Duitman et al. 2007). The horizontal transfer of iturin operon in *Bacillus subtilis* 168 together with a functional *sfp* gene and the pleiotropic regulator *degQ* allowed conversion of this strain to an efficient iturin producer (Tsuge et al. 2005). Recently, the combined use of low temperature, isoleucine addition and overproducing mutant strains with P_{xyl} instead of P_{myc} driving the mycosubtilin operon allowed us to synthesise more than 900 mg l^{-1} of the most active mycosubtilin isoform against *Candida* sp. (Fickers et al. 2009).

6.4 New Bioprocesses for Continuous Production and Extraction of Lipopeptides

The different strategies that have been used for the fermentation process and extraction methods for the production of lipopeptides are discussed.

6.4.1 Quantitative Evaluation of Surfactin Concentrations

Several indirect techniques based on surfactin properties, such as measurement of surface tension or haemolytic activities (Jacques et al. 1999), are used for

quantitative evaluation of surfactin. These techniques are mainly dependent on the degree of purity of the tested samples and can thus only give a qualitative idea about the presence of surfactant or relative idea about their concentrations. Analysis by high performance liquid chromatography is the main accurate technique for surfactin evaluation, but it needs to be carefully conducted (Razafindralambo et al. 1993). As surfactin precipitates at pH below 6, samples should be stored at higher pH. In several cases, high salt or organic compound concentrations can also lead to precipitation during sample freeze-thawing. If pre-step purification by solid-phase extraction is recommended, its working conditions depend of the nature of the pre-column used, and it has to be carefully tested before being routinely used to avoid the partial loss of the surfactin present in the samples.

6.4.2 Purification of Surfactin

Surfactin was first extracted from culture broth by acidic precipitation (HCl) followed by extraction with methanol or other organic solvents. Purification was completed by chromatographic procedures (on silica gel or by reversed phase chromatography). Other methods were then proposed to purify and concentrate the lipopeptide: a two-phase extraction (Drouin and Cooper 1992), an ultrafiltration method (Mulligan and Gibbs 1990; Isa et al. 2008), a solid-phase extraction (Razafindralambo et al. 1993; Montastruc et al. 2008), a liquid membrane extraction (Dimitrov et al. 2008) or different combined methods (Chen et al. 2007, 2008).

6.4.3 Bioprocesses for Lipopeptide Production

Three main types of bioprocesses were developed for lipopeptide production: solid-state fermentation, foam fractionation and membrane bioreactors. Solid state fermentation has been suggested for a long time by Japanese researchers, as *Bacillus subtilis* can easily grow on different food processing wastes. They showed a high level of surfactin production (2 g kg^{-1} of wet weight) (Ohno et al. 1995b). The technique of foam fractionation was first suggested by Cooper et al. (1981). It offers the double advantage of continuous in situ removal of produced surfactin from the fermentation broth together with the prevention of any possible feedback inhibition. It was then developed by Davis et al. (2001). We also applied this strategy for the production of mycosubtilin. An overflowing exponentially fed batch process (OEFBC) was so defined, which allowed a continuous extraction of the mycosubtilin with high efficiency (Guez et al. 2007). With the same idea of developing a continuous process for surfactin and fengycin extraction, a membrane bioreactor was tested. The use of a membrane to ensure the oxygen transfer in bubbleless conditions allowed to avoid foam formation, leading to the development of a membrane bioreactor with cell recycling by microfiltration and lipopeptide concentration by ultrafiltration (Coutte et al. 2010b).

6.5 Conclusion

The actual level of knowledge of the biosynthesis of lipopeptide from *Bacillus subtilis* and its regulation mechanism allows to develop different strategies to overproduce the main active compounds and to reach yields that are compatible with industrial development of such compounds.

7 Industrial Applications: Dream and Reality

In this section, we summarise the main potential applications of surfactin developed in literature and the commercially existing products. Figure 4 gives an overview of application sectors of surfactin.

7.1 Main Applications

Several recent reviews summarise the high interest of biosurfactant for different application fields (Singh et al. 2007): food (Nitschke and Costa 2007), petroleum recovery (Sen 2008), environmental (Cameotra and Makkar 2010; Mulligan 2009), biomedical (Rodrigues et al. 2006) and cosmetics (Kanlayavattanakul and Lourith 2010). During its long history, surfactin has been first studied for its potential

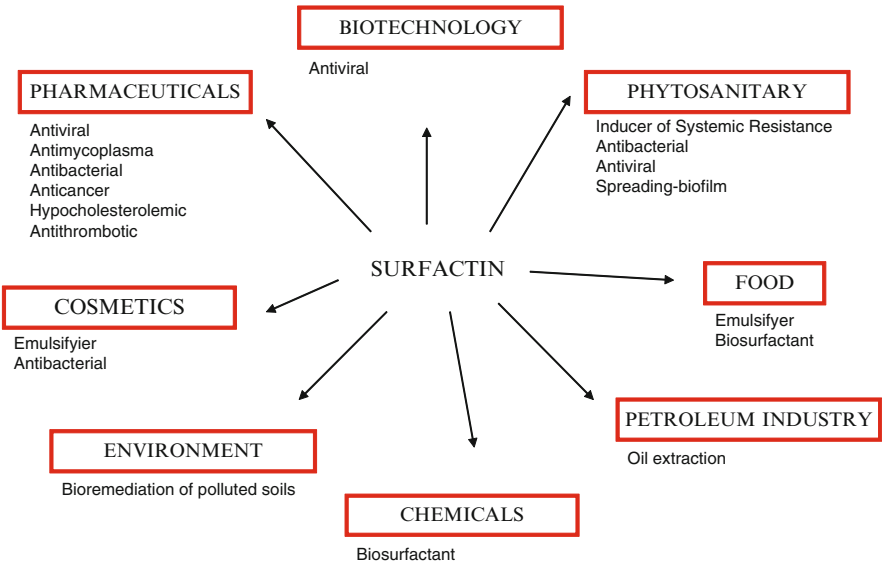

Fig. 4 Application sectors and exploited properties of surfactin

pharmaceutical applications (antibacterial, antitumoral and hypocholesterolemic activities). Its biosurfactant properties were also highlighted, and its application for example in petroleum industry was considered. The discovery at the end of 1990s of its antimycoplasma and antiviral properties led to the proposal of its use to ensure the safety of pharmaceutical or biotechnological products. The presence of several lipopeptides in different Asian (detection of several µg of surfactin/100 g of Natto wet weight, Sumi et al. 2000) or African (presence of mycosubtilin in Netetu, N'dir et al. 1994) fermented food products or its secretion by strains isolated from these products (Cho et al. 2009) allows for consideration of their application in the food sector. More recently, its ability to induce systemic resistance in plants and its implication in the spreading of the cells and thus the rhizosphere colonisation could open new fields of applications as phytopharmaceutical products (Ongena and Jacques 2008).

7.2 Conclusion

More than 50 publications published in 2009 are referenced by Scopus with the keyword "surfactin" showing the high interest in this compound, which remains the most studied lipopeptide produced by *Bacillus* spp. Despite this high number of scientific publications and patents, industrial surfactin applications still remain quite limited. Sold by SIGMA and SHOWA DENKO Co for analytical or laboratory purposes, the compound is also available in several Japanese cosmetics products. Production costs and also suspected toxicity (From et al. 2007) are probable reasons why these compounds are not yet commonly used. Recent development of overproducing strains and upscalable bioprocesses could solve the problem of cost efficiency. Toxicological and ecotoxicological studies are now needed to confirm the low toxicity (Hwang et al. 2009) of a compound which is probably consumed every day by thousands of people.

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